

**EFFECT OF STRUCTURAL MODIFICATIONS OF ANTHRACYCLINE
AGAINST DRUG-SENSITIVE AND RESISTANT CANCER CELLS**

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ABSTRACT

Purpose: Many anthracycline anticancer drugs contain carbohydrate moieties as a part of their chemical structure. Research studies have suggested that the sugar moiety of these anthracyclines play an important role in determining the biological and pharmacological activities of the drug. The clinical application of anthracycline antibiotics, daunorubicin (DNR) and doxorubicin is limited by the development of multidrug resistance (MDR) in cancer therapy. To overcome multidrug resistance, we synthesized daunorubicin derivatives with disaccharides, monosaccharide bearing azido (**d9**) and triazole groups (compounds **d10-d13**), monosaccharide with uncommon (DNR-1 to DNR 6, and aglycon compound (DNR-A). **Methods:** The anticancer activity of these compounds was examined in drug-sensitive leukemia K562 cells and drug-resistant K562/dox cells by MTS assay. MTS in the presence and absence of CsA, a potent inhibitor of P-glycoprotein, were conducted to measure drug uptake and efflux mediated by P-gp. **Results:** Compounds with various terminal 2,6-dideoxy sugars (**d1**, **d3**, **d5**, and **d8**) showed 30 to 60-fold higher anticancer activity than compounds with terminal 2-deoxy- or 6-deoxysugar (compound **d6** and **d7**). This suggests that 2''-OH or 6''-OH groups in terminal sugar decrease its anticancer activity. Compound (**d9**) with azido deoxysugar showed potent anticancer activity in drug-sensitive K562 cells (IC_{50} of 0.075 μ M). Compared to DNR, compound (**d9**) is more active than DNR against K562 drug-resistant cells. The drug resistance index (DRI) value of compound (**d9**) is 10-fold less than that of DNR. P-gp inhibitor CsA did not change cell killing effects of compound **d9** at 1 μ M concentration, while it significantly increased DNR's cell killing effect by more than 2-fold. These results indicate that compound (**d9**) may no longer be a P-gp

substrate and overcome P-gp mediated drug resistance. Compared to daunorubicin derivatives with various uncommon sugars, the aglycon compound DNR-A showed 70- to 100-fold lower cytotoxicity, which suggests that the sugar moiety for anthracyclines is important for cytotoxicity. **Conclusion:** Modifications of sugar structures of daunorubicin significantly changed its anticancer activity. Daunorubicin derivative with an azido in the sugar structure may avert the recognition by P-gp and overcome P-gp-mediated drug resistance.

INTRODUCTION

Anthracyclines are natural products antibiotics with potent anticancer activity. Doxorubicin and daunorubicin are among the first to be clinically approved in the late 1960s and early 1970s. Doxorubicin has a wide range of anticancer activity against leukemia, breast cancers and sarcomas while daunorubicin is very effective in the treatment of leukemia. A typical problem which is facing chemotherapy is the existence of multidrug resistance characterized by the over-expression of P-glycoprotein (P-gp). P-gp pumps drugs out from cancer cells, reduces intracellular cell concentration, and induces drug resistance.

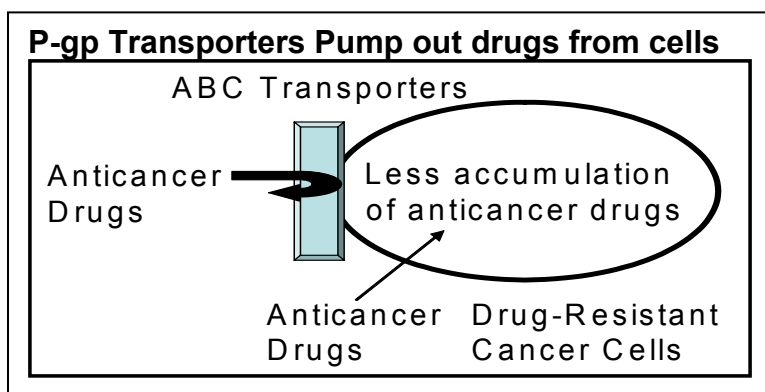


Figure 1. P-gp exports drug from cells and induces multidrug resistance

HYPOTHESIS

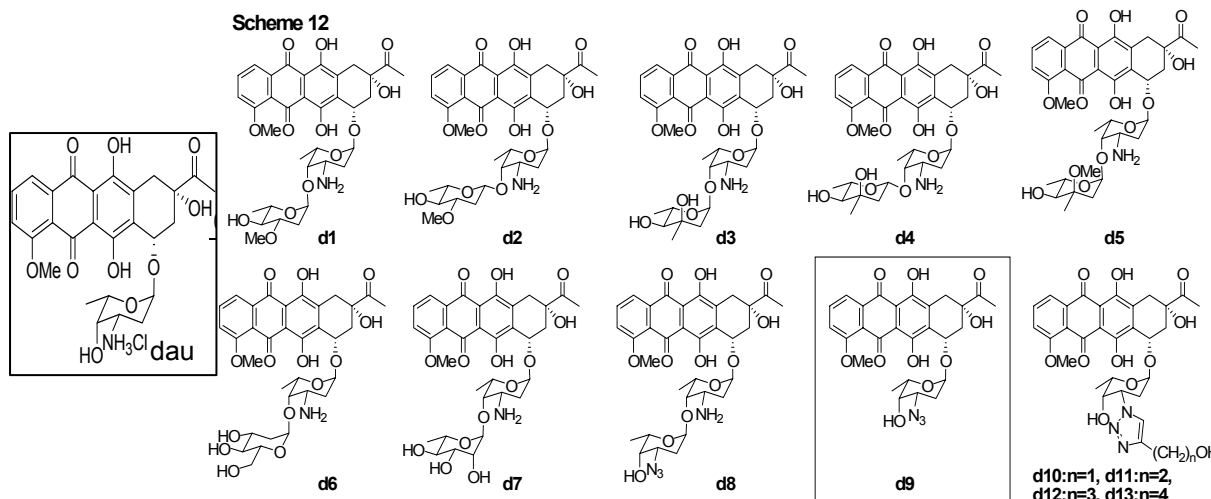
We hypothesize that direct modification of sugar moieties of daunorubicin may avert recognition by P-gp, increase drug intracellular concentration in cancer cells, and thus overcome drug resistance.

MATERIALS AND METHODS

Analog of daunorubicin was synthesized (D1-D8) with disaccharides of terminal 2,6-dideoxy sugar or 2-deoxy- or 6-deoxy-sugar (Figure 2). D9 was a lead compound with an azido group in the sugar structure. The other analogs (D10-D13) had their 3' position replaced with a larger triazole group. The cytotoxicity of the newly synthesized analogs of daunorubicin, was tested using MTS [3-(4,5-dimethylthiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay in drug-sensitive leukemia K562 and drug-resistant K562 (K562/dox) cells, and colon cancer (SW620) cells. The positive control with daunorubicin and a negative control with no drugs were included.

Cytotoxicity of daunorubicin analogues by MTS assay: Drug-sensitive K562 and drug-resistant K562/Dox cells (2,000-10,000) were seeded in 96 well plates in RPMI-1640 and incubated for 24 hours. The exponentially growing cancer cells were incubated with various concentrations of compounds for 72 hours at 37 °C (5% CO₂, 95% humidity). After 72 hours incubation, tetrazolium[3-(4,5-dimethylthiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, 2 mg/ml) and phenazine methosulfate (PMS, 25 µM) were mixed and added directly to the cells. After incubation for 3 hrs at 37 °C, the absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm. The IC₅₀ values of the compounds for

cytotoxicity were calculated by WinNonlin software from the dose-response curves. The standard deviation calculated from six experimental replicates was calculated and used as the error bar for the graph.



Daunorubicin and its analog with disaccharide and azido group

Drug resistance assay of daunorubicin analogues: K562/Dox leukemia cells (2000-10,000) were seeded in 96-well plates in RPMI-1640 and incubated overnight. The cells were pretreated with 5 μ M Cyclosporine A (CsA) for 10 minutes. Then the synthesized compounds (1 μ M) were added. After 72 hours, tetrazolium[3-(4,5-dimethylthiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, 2 mg/ml) and phenazine methosulfate (PMS, 25 μ M) were mixed and added directly to the cells. After incubated for 3 hrs at 37 $^{\circ}$ C, the absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm. The Drug Resistance Index (DRI), which is the ratio of IC_{50} in K562/DOX verse IC_{50} in K562, was calculated.

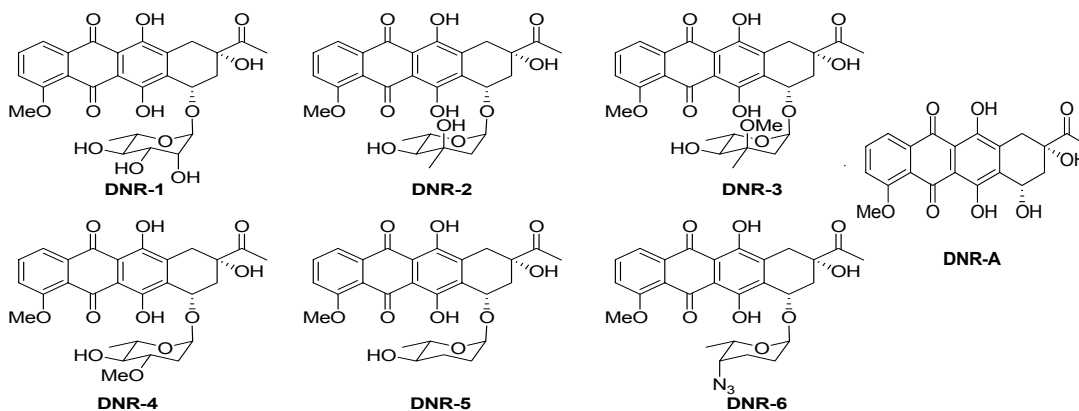


Figure 3. Daunorubicin analogues with uncommon sugar

RESULTS AND DISCUSSION

α - linkage between disaccharides of daunorubicin analogues provides better activity than β -linkage

Compound with α -linkage between two sugar units (**d3**) showed 35-fold higher anticancer activity than compound with β -linkage (**d4**) (Fig. 4). This indicates that α -linkage configuration was the mostly likely preferred conformation for the sugar moiety, and therefore modification will be geared towards improving α - linkage between sugar structures.

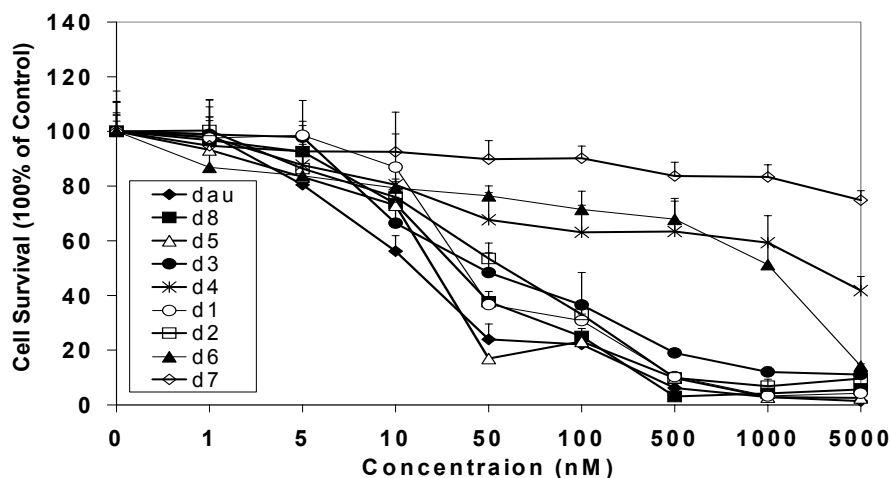


Figure 4. Anticancer activity of disaccharides Daunorubicin

Dideoxy sugar structure in daunorubicin analogues showed higher anticancer activity than deoxy sugars

Compounds with various terminal 2,6-dideoxy sugars (**d1**, **d3**, **d5**, and **d8**) showed 30 to 60-fold higher anticancer activity than compounds with terminal 2-deoxy- or 6-deoxysugar (**compound d6 and d7**) (Fig. 4). This suggests that 2''-OH or 6''-OH groups in terminal sugar decrease its anticancer activity.

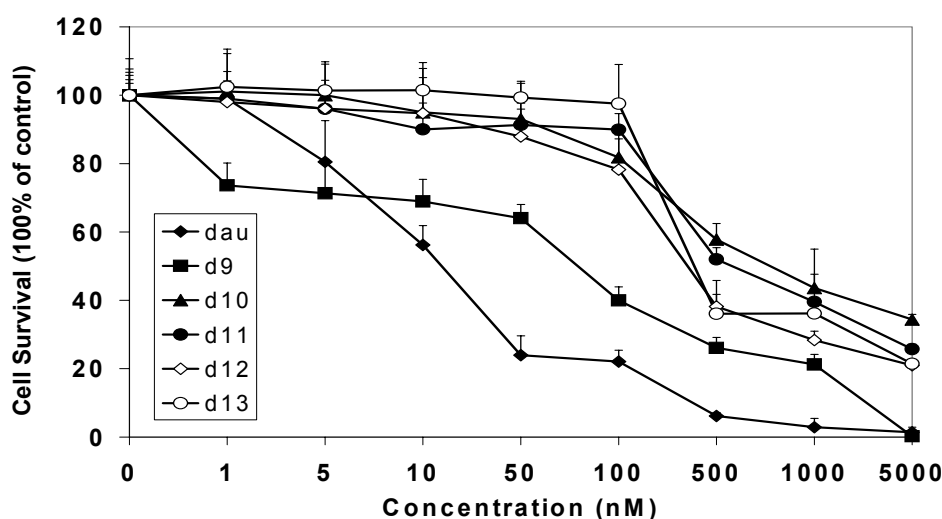


Figure 5. Anticancer activity of Monosaccharide Daunorubicin

-NH₂ replacement of daunorubicin changes its activity

When -NH₂ in the primary sugar of daunorubicin changed to larger moieties with triazole (compound **d10-d13**), its anticancer activity significantly decreased by 16 to 30-fold (Figure 5). However, when the -NH₂ of daunorubicin changed to -N₃ (compound **d9**), its anticancer activity slightly decreased. These data indicate that modification of the first sugar moiety will result in significant change in its activity.

Azido group in sugar moiety of daunorubicin showed better activity against drug-resistant leukemia cells.

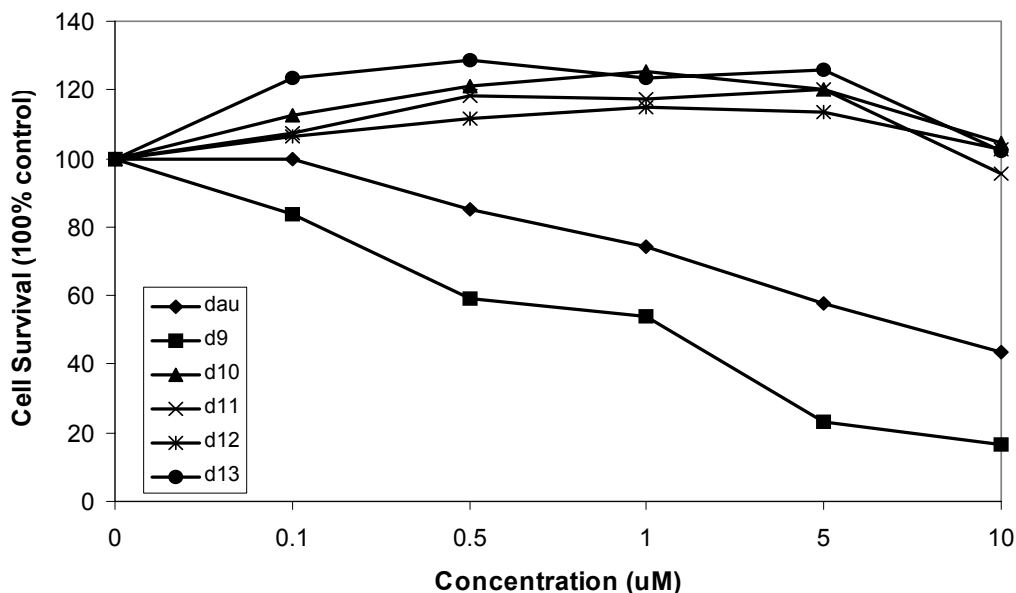


Figure 6. Anticancer activity of Daunorubicin analogs in drug resistant leukemia K562/dox

By sugar modification, we have successfully found a lead compound (**d9**) to overcome drug resistance. Its activity was tested in drug-resistant leukemia cells (K562/R). These cells have expressed 4 to 1000- fold higher P-gp expressions compared to parent drug-sensitive cancer cells. Compound **d1**, **d5**, and **d9** showed improvements in overcoming drug resistance. Their drug resistance index (DRI, ratio of IC₅₀ in drug-resistant cells over IC₅₀ in drug-sensitive cells) decreased by more than 2 to 10-fold compared to daunorubicin (Table 1, Figure 6). Very surprisingly, the DRI of compound **d9** decreased to 13 compared to DRI of daunorubicin with value of 139.

Table 1 Drug resistance index data.

#	IC ₅₀ in K562 (nM)	IC ₅₀ in K562/R (uM)	DRI (Ratio)
d1	39.5	2.1	53
d2	45.8	4.6	101
d3	44.3	3.6	81
d5	21.0	1.3	65
d8	31.3	2.4	79
d9	75.4	1.0	13
dau	15.6	2.2	139

Compound (d9) is no longer a P-gp substrate and may overcome drug resistance

To further investigate the mechanism of compound (**d9**) in overcoming drug resistance, we tested P-gp involvement for drug-resistance profile of our compounds. Because one of the mechanisms for drug resistance in cancer cells is due to the export of anticancer drug from cancer cells by P-gp transporter, and thus decreasing drug intracellular concentration. If a drug is a P-gp substrate, P-gp inhibitor (cyclosporine, CsA) will inhibit the drug export by P-gp, increase drug intracellular concentration, and thus increase drug cytotoxicity.

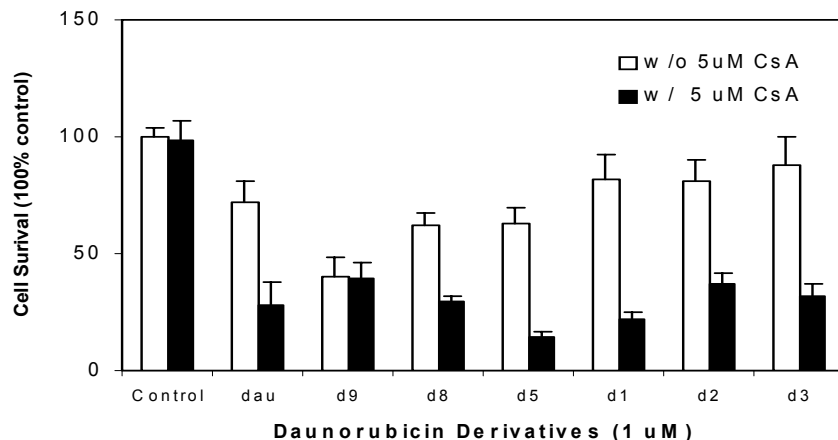


Figure 7. Cytotoxicity of Daunorubicin and compounds (D1, D2, D3, D5, D8, and D9) in drug resistant K562/dox cells in the absence and presence of CSA

As shown in Figure 7, when CsA is used to inhibit drug export by P-gp, daunorubicin and most of its derivatives (compound **dau**, **d1**, **d2**, **d3**, **d5**, and **d8**) showed more than 2-fold increase for their cell killing effect. This indicates that these compounds are still P-gp substrates and may still exhibit drug resistance. However, CsA did not change the anticancer activity of our lead compound **d9** in K562/R (Figure 7). This data

suggests that compound **d9** is no longer a P-gp substrate and may therefore overcome drug resistance in cancer cells.

Daunorubicin derivatives with uncommon sugars

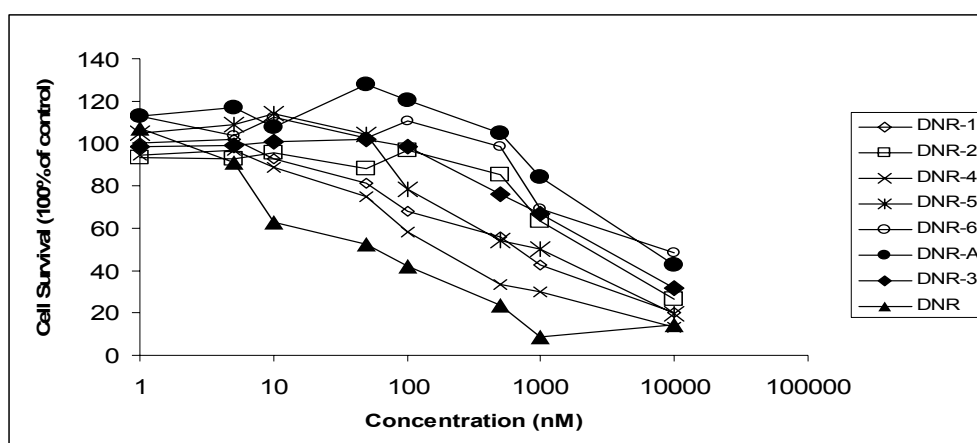


Figure 8. Anticancer activity of analogs in SW620

The results showed that the aglycon **DNR-A** exhibited 70- to 100-fold lower cytotoxicity than daunorubicin derivatives with various uncommon sugars. This suggests that sugar structure in daunorubicin plays a critical role in determining its anticancer activity.

Compound **DNR-4** with 3'-OMe 2,6-dideoxy sugars showed very potent cytotoxicity with IC_{50} of 104 nM (Figure. 8). Importantly, compared to compounds **DNR-2** and **DNR-3** (with *axial*-3'-OMe or *axial*-3'-OH group), **DNR-4** (with an *equatorial*-3'-OMe group) showed 10 to 20-fold higher anticancer activity. This suggested that the *axial*-3'-substituent in sugar (such as in compounds **DNR-2** and **DNR-3**) may interfere daunorubicin binding to DNA.

Compound 1 and 5 with equatorial-4'-OH showed similar activity, while **DNR-6** (substituted 4'-OH with axial-4'-N₃ in the sugar moiety), which is similar to aglycon DNR-A, lost its cytotoxicity (Fig 8). This suggests that 4-OH in the sugar may also be important for sugar containing anthracycline as an anticancer agent. Further SAR studies are required to clarify this finding.

CONCLUSIONS

In summary, daunorubicin derivatives with α -linkage between two sugar units (**d3**) showed 35-fold higher anticancer activity than compound with β -linkage (**d4**). This indicates that α -linkage configuration was the mostly likely preferred conformation for the sugar moiety. Compounds with various terminal 2,6-dideoxy sugars (**d1**, **d3**, **d5**, and **d8**) showed 30 to 60-fold higher anticancer activity than compounds with terminal 2-deoxy- or 6-deoxysugar (compound **d6** and **d7**). This suggests that 2''-OH or 6''-OH groups in terminal sugar decrease its anticancer activity. Daunorubicin analog with 3'-azido (**d9**) exhibited potent anticancer activity in both drug-sensitive and drug-resistant leukemia K562 cells. Compound (**d9**) showed significantly better anticancer activity than DNR in drug-resistant K562/Dox cells with 10-fold lower drug resistance index. P-gp inhibition by CsA confirmed that compound (**d9**) is no longer a P-glycoprotein (P-gp) substrate; therefore, compound (**d9**) may overcome P-gp-mediated drug resistance in cancer cells. Modifications of sugar moiety of the lead compound (**d9**) provide a practical approach to avert the recognition P-gp transporters, and thus overcome drug resistance. We are continuing to investigate the biological activities of compound (**d9**) in xenograft mouse models in vivo. Chemical modifications to optimize this series of compounds for SAR studies and improve their activity are currently in progress. Compound DNR-A

showed the lowest cytotoxicity (70 to 100-fold) compared to DNR, which further suggest that sugar moiety in daunorubicin is very important for its anticancer activity.

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